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Cre/*lox*P-mediated adenovirus type 5 packaging signal excision demonstrates that core element VI is sufficient for virus packaging

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Abstract

Previous analyses have demonstrated that packaging of the adenovirus type 5 (Ad5) genome is dependent on at least seven *cis*-acting elements, called AI to AVII, which are located in the left-end region of the genome. These elements have different packaging efficiencies, and without AI through AV, viral DNA cannot be packaged. Here we report the identification of the *cis*-acting Ad5 packaging domain in vivo by using the Cre/*lox*P system. We found that an adenoviral DNA fragment (nt 192 to nt 358), which includes elements AI to AV, is excised by Cre recombinase and packaged into capsids. Furthermore, this mutant adenovirus replicated so efficiently by repetitive propagation that its purification by CsCI equilibrium gradient was possible. This study clarified that the region from nt 358 to nt 454 on the viral genome is sufficient for packaging. Recently, the helper-dependent adenoviral vector (HDAd) construction system has been developed for the purpose of gene therapy. This system uses a helper virus with two parallel *lox*P sites flanking the packaging signal. This region is eliminated by Cre-mediated excision, which prevents helper virus packaging. Our data provide useful information regarding factors affecting efficient elimination.

Introduction

Packaging of the adenovirus type 5 (Ad5) genome occurs in a polar fashion from left to right and is mediated by a *cis*-acting packaging domain located within the 380 bp at the left end of the genome (Hammarskjold and Winberg, 1980; Hearing et al., 1987; Robinson and Tibbetts, 1984). Detailed analysis of the packaging domain by deletion as well as linker scanning mutagenesis demonstrated that it consists of at least seven core elements termed AI through AVII from the left terminus (Fig. 1). Because these elements contain an AT-rich sequence, they are called A repeats. Although similar sequences are present in AI–VII, the ability of each A element to support DNA packaging is different (Grable and Hearing, 1990, 1992). Among the seven elements, the functionally most efficient packaging elements are AI, II, V, and VI, each of which exhibits the consensus motif 5′-TTTGN$_8$CG-3′ (Schmid and Hearing, 1997). More interestingly, this consensus motif is conserved across a number of Ad serotypes. With the exception of AVI, the A elements are able to package viral DNA at different efficiencies and each works independently. There is a hierarchy of functional importance even within the group of the most efficient packaging elements, with AVI as the weakest element followed by AII, AV, and finally AI as the most functionally dominant A repeat. At least six copies of the AVI element are necessary to package viral DNA that lacks the other packaging elements. This mutant virus has a more than 100-fold reduction in growth relative to the wild-type virus, but AVI is easily amplified and this results in an improved packaging efficiency (Schmid and Hearing, 1997, 1998).

While the nature of adenovirus has been studied in detail, adeno-based vectors are developed for gene therapy. The first-generation adenovirus vector that was developed and widely used has an alteration in the viral E1/E3 regions, allowing it to carry a therapeutic gene (Akli et al., 1993; Drazan et al., 1995; Feldman et al., 1996; Kimura et al.,

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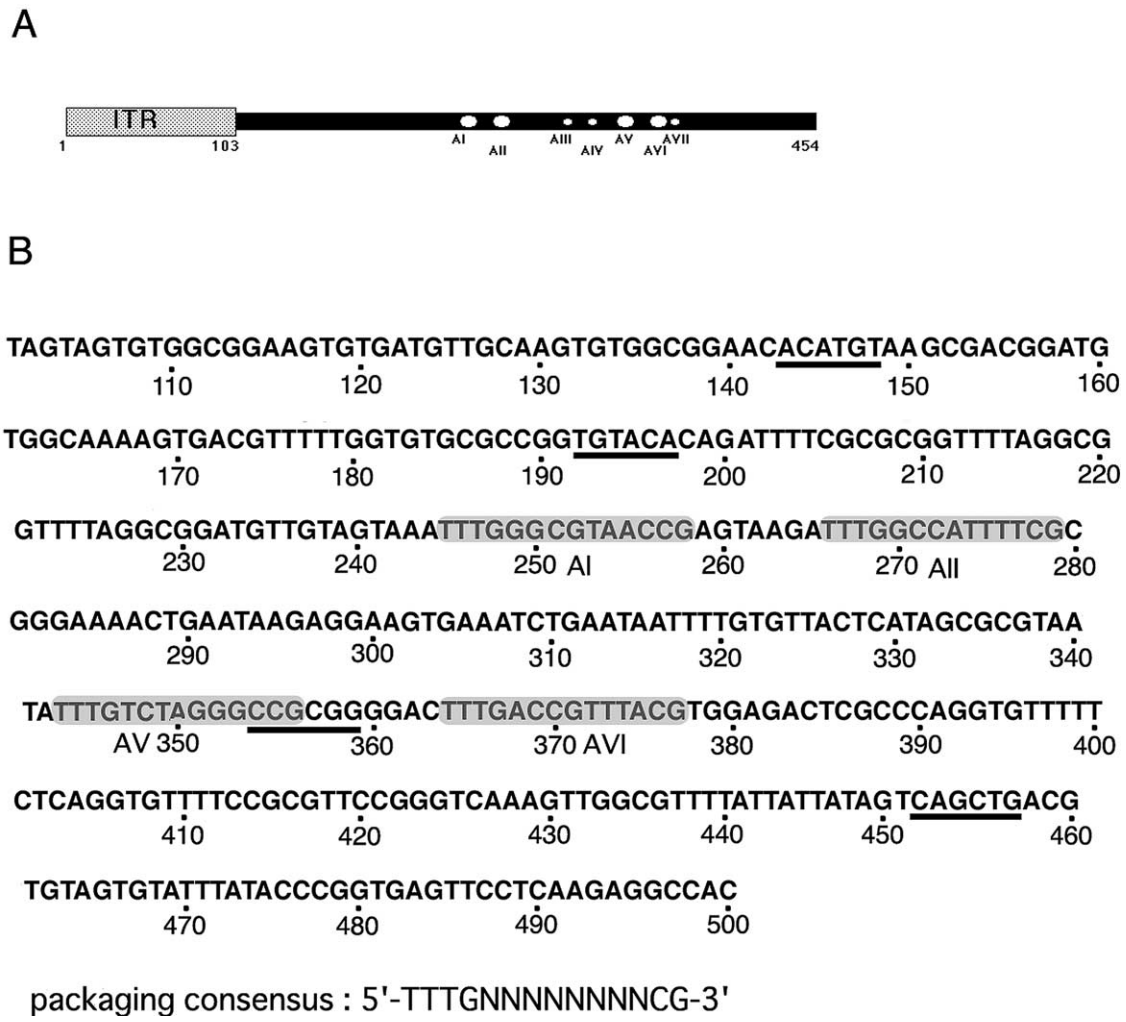

Fig. 1. The adenovirus type 5 left-end and packaging domain (Schmid and Hearing, 1997). (A) The schematic drawing shows the ITR and the packaging region. The packaging elements (AI through AVII) are represented by ovals. Major elements (AI, AII, AV, and AVI) are indicated by large ovals. The ITR is represented by a shaded rectangle. (B) Nucleotide sequence of the Ad5 packaging domain. Numbers correspond to nucleotides relative to the left-end terminus. Colored circles indicate the four major elements. *lox*P sites are inserted into the underlined positions.

2001; Le Gal La Salle et al., 1993; Petrof et al., 1996; Rothmann et al., 1996; Wickham et al., 1996). Because of the deletion in the viral E1 regions, this virus is permissive only in 293 cells that stably express E1 proteins. However, it has some practical defects. There is an immune response against the virus or the transduced cells because of the leaky expression of some of the viral genes. In addition, it has a limited cloning capacity (~8 kb). Recently, a helper-dependent vector (HDAd) has been developed to overcome these defects. HDAd contains only the *cis*-acting elements required for viral DNA replication and packaging, mainly the inverted terminal repeat (ITR) sequences and the packaging signal (ψ). Therefore, HDAd is a less immunogenic vector with an insert capacity of up to 37 kb (Clemens et al., 1996; Kochanek et al., 1996; Mitani et al., 1995).

In constructing the HDAd, the first-generation adenovirus with two parallel *lox*P sites encompassing ψ is used as a helper virus to provide all necessary proteins in trans for packaging of the vector DNA with ITR/ψ and the therapeutic gene. The Cre/*lox*P system is a recombination system derived from bacteriophage P1. The 38-kDa Cre recombinase protein binds to the two 34-bp *lox*P sequences and catalyzes precise recombination between the two *lox*P sites. When recombination occurs between parallel sites, the intervening sequence is excised (Lu et al., 1989; Sauer and Henderson, 1989). Since the helper virus is a first-generation adenovirus with two parallel *lox*P sites flanking the packaging signal, when a 293 cell line expressing Cre recombinase is infected by this helper virus, the helper virus becomes unpackagable due to Cre-mediated packaging signal excision. To increase the titer of the HDAd helper virus, coinfection of Cre-expressing 293 cells with low-titer HDAd must be repeated (Parks et al., 1996; Morsy, 1998 #19). Therefore, if a bit of helper virus is packaged during each propagation, eventually helper virus contamination will no longer be negligible. Complete inhibition of helper

virus production is very critical to suppress helper virus contamination during HDAd preparation. Therefore, we analyzed which portion of the Ad5 packaging domain is sufficient for virus encapcidation and purification under repetitive propagation conditions. In this study, we found that the region from nt 358 to nt 454, which includes the weakest of the four major elements (AVI), can confer an ability sufficient for packaging the viral genome even without amplification of the region.

Results

Complete excision between the two loxP sites of the adenovirus genome and vigorous replication in Cre-293

We constructed three kinds of first-generation adenoviruses having two parallel *lox*P sites around the left end of the genome, AdASw, AdBSa and AdBSw, which had titers of 7×10^{10}, 4×10^{10}, 3×10^{10} plaque forming units/ml (PFU/ml) (Fig. 2A). When AdASw, AdBSa, and AdBSw were used to infect Cre-293 cells, fragments containing nt 143–454, 192–358, and 192–454 were excised, respectively. Because these viruses were derived from a first-generation adenovirus, they lacked the region from nt 455–4500 and carried a 2.5-kb luciferase expression cassette and 3.7-kb λ-phage fragment instead. We confirmed the complete excision by Southern blotting and/or polymerase chain reaction (PCR), because interpretation of the results is confusing if the intervening sequence is not completely excised. Cre-293 cells were infected with each virus for 1 h, and cells were washed three times with PBS. Viral and cellular DNA was harvested using the standard method at 8, 24, and 48 h postinfection. To remove the free virus present in the culture medium from the cells, cells were collected by centrifugation and washed repetitively with PBS. The left-end region of each respective virus was amplified by PCR using the ITR and λ-primers. Forty-eight hours after infection, excision was complete, and only the small PCR product was detected in each viral DNA preparation (Fig. 2B). Because these results only prove complete excision, we analyzed the state of the viral DNA in Cre-293 at each time point by Southern hybridization using a λ-specific probe. As expected, the excision was complete 24 h after infection. We confirmed that the recombinant viruses replicated rapidly for 48 h postinfection. In addition, viral DNA was able to be detected after 24 h, but was not at 8 h postinfection (Fig. 2C). To detect the unrecombinated viral DNA for the enhanced sensitivity, we performed PCR and then Southern hybridization by using the ITR as the probe. Even in the overexposure of the membrane to the film, we could not detect any band indicating unrecombinated viral DNA (Fig. 2D). If these three kinds of recombinated viruses replicate with the different efficiency, interpretation of the results becomes difficult. So we quantitated these viral DNAs at 48 h postinfection by the competitive PCR. As we had already proved that the preparated DNA did not contain unrecombinated DNA, pAdex-ASw-lox-luc λ was added as an internal standard in the PCR reaction. The left-end region was amplified by using the ITR and λ-primers. This competitive PCR showed that all viruses replicated with same efficiency after the recombination (Fig. 2E). These results demonstrate that the Cre recombinase expressed in Cre-293 cells could recognize viral *lox*P sites and excise the intervening region completely, and all resultant viruses were able to replicate vigorously and to the same degree.

Deletion of nt 192 to nt 455 resulted in a nonviable virus, but a virus with a deletion from nt 192 to nt 358 was packaged and viable

After complete excision between two *lox*P sites in Cre-293 was confirmed, we analyzed whether the packaged virus appeared after Cre-mediated recombination by carrying out plaque assays on 293 cells. The titers of AdASw and AdBSw remained constant even after repetitive propagation at 10^6–10^7 PFU/ml. Because the medium was not changed after the infection, it is probable that uninfected viruses in the culture medium are able to form plaques. On the other hand, the titer of AdBSa increased rapidly after the fourth, fifth, and sixth propagations (P4, P5, P6). During these propagations, the viral titer increased almost five times, and after the seventh propagation (P7), the viral titer reached 10^9 PFU/ml. This indicates that the deletion from nt 192 to nt 358 is not sufficient to inhibit the encapsidation of viral DNA (Fig. 3) or a second site mutation(s) has occurred that results in a revertant virus that grows better. To address this, we used the virus stocks at P1, P4, and at P6 for single-step growth curves. When 4×10^7 PFU of P4 and P6 virus was used to infect Cre-293 cells in a 60-mm-diameter dish, the infectious virus yield at these different propagations was determined by a plaque forming assay. The yield of virus using P4 stock was 3×10^8 PFU and that of virus using P6 stock was 2×10^8 PFU. The yield of virus using P4 stock vs P6 was almost same and 9×10^5 PFU of P1 was also used to infect 60-mm-diameter dish Cre-293 cells. The yield of virus using P1 stock became 3×10^6 PFU. After the single-step growth each virus could amplify with three to seven times amplification efficiency. These reults indicate that the stock is simply amplifying over time with no genetic alterations of the virus; the possibility that a second mutation has occurred to rescue the packaging defect is excluded (Table 1).

Moreover, we were apprehensive about the appearance of wild-type adenovirus due to the rearrangements between the left-end portion of test virus and the E1 region in the chromosome of Cre-293 cells; cell lysates from P7 were used to detect wild-type adenovirus on A549 cells. Even after repeated propagation, however, wild-type adenovirus or any replication-competent virus was not detected.

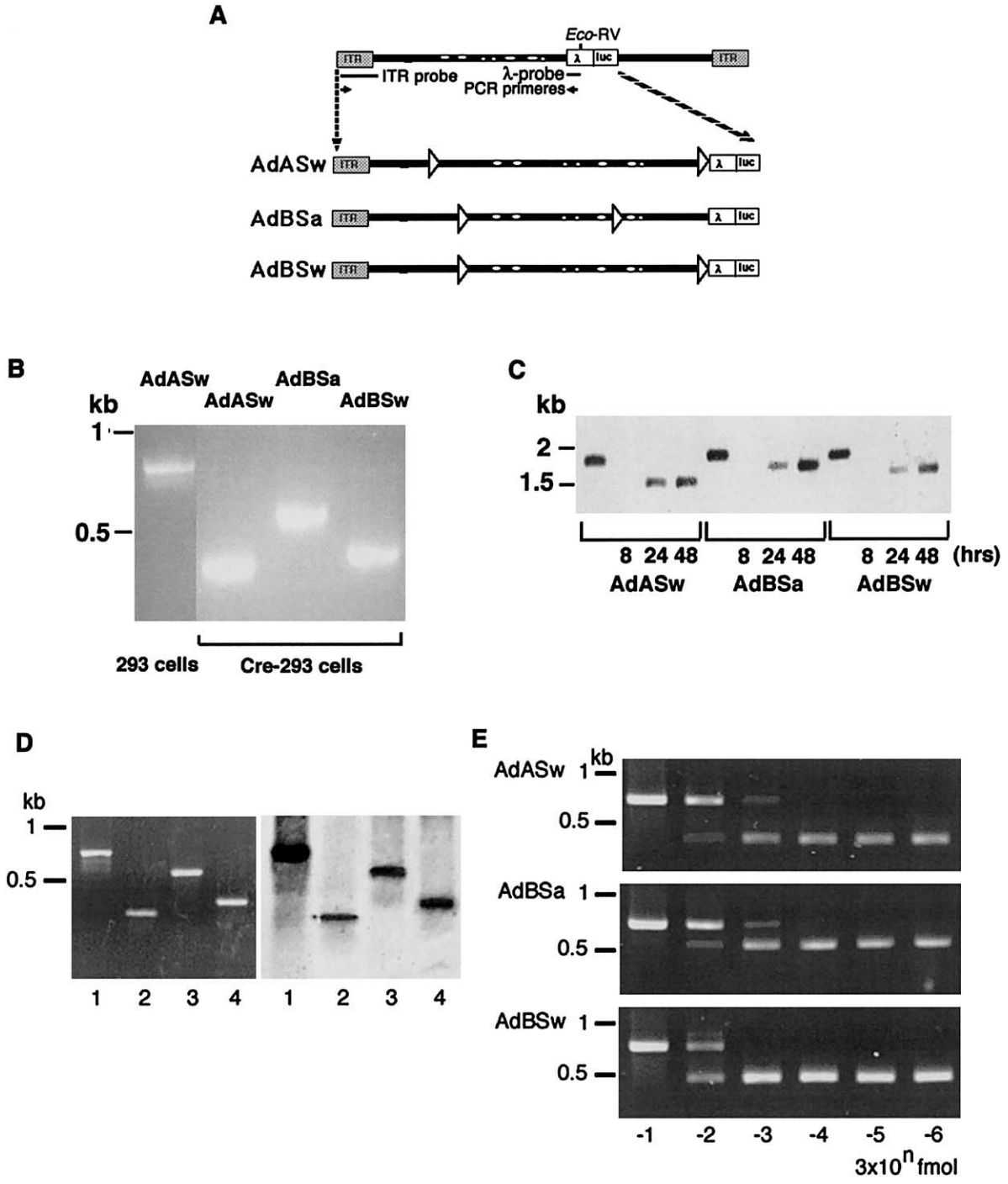

Fig. 2. Cre-mediated complete excision of viral packaging *cis*-elements. (A) The structures of adenoviruses used in this study. Viral left-end structures are depicted. The E1 region of the virus is replaced by a 6.2-kb DNA fragment containing a 3.7-kb λ-fragment and a 2.5-kb luciferase expression cassette. The triangle indicates the *lox*P site. (B) The result of PCR amplification of the left-end region of the virus genome 48 h after infection of either 293 cells or Cre-293 cells. Primer positions are depicted in (A). No higher molecular weight band was detected in the three lanes on the right. (C) Complete excision was confirmed by Southern blot analysis by using the λ-probe as depicted in (A). Each virus was used to infect Cre-293 cells in 60-mm-diameter culture dishes, and the viral DNA and cellular DNA were collected from the cells 8, 24, and 48 h later. Two micrograms of each preparation was digested with *Eco*RV, separated on a 1% agarose gel, transferred to Hybond-N+ (Amersham), and probed using an alkaline phosphatase-labeled λ-probe. *Eco*RV-digested viral and cellular DNA generated 48 h after infection of 293 cells is loaded in the left-most lane in each bracket. (D) PCR/Southern blot analysis to detect the unrecombinated virus DNA for enhanced efficiency. PCR was performed as mentioned in (B), and then its Southern blot was probed to labeled ITR probe as depicted in (A). No unrecombinated virus DNA was detectable. Lane 1: AdASw grown in 293 cells for 48 h. Lane 2, lane 3, and lane 4 correspond to AdASw, AdBSa, and AdBSw grown in Cre-293 for 48 h, respectively. Left panel shows agarose-gel separation. Right panel shows the result of Southern blot analysis. (E) Quantitative analysis of virus DNA in 48 h postinfection by the competitive PCR. Virus DNA and the internal standard were amplified by PCR using a pair of primers mentioned in (B). pAdex-ASw-lox-lucλ was added as an internal standard in the PCR. The numbers −1 to −6 mean an exponent of 3×10^n fmol/reaction. All viruses replicated with same efficiency after the recombination in Cre-293 cells.

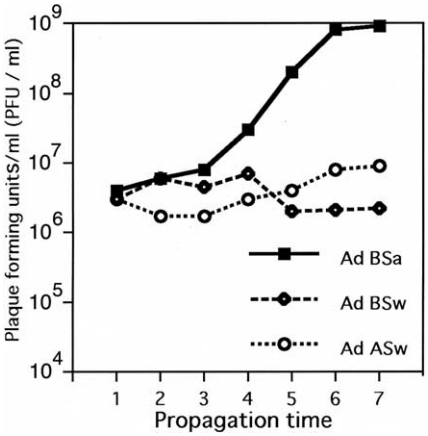

Fig. 3. Amplification of each virus in Cre-293 cells. Packaged virus was collected from Cre-293 cells and culture medium 48 h postinfection by three freeze-thaw cycles. The virus titer was determined by plaque assays on 293 cells. The Cre-293 cells were infected with various amounts of crude lysates that contain the recombinant and uninfected viruses and overlaid with medium containing 0.5% agarose. The dishes were observed every 2 days for 2 weeks. The virus titer of crude lysates from AdBSa infection increased rapidly after the fourth propagation, but the other were constant.

Large-scale production of a mutant virus with a deletion from nt 192 to nt 358

It has been reported that the minimal packaging element is AVI, but a mutant virus containing a hexamer of AVI in place of the packaging domain exhibits precise amplification of AVI for more efficient packaging. We tested whether it is possible to purify the excised virus from the large-scale preparation without packaging element amplification. The virus was purified by CsCI density gradient purification, and after centrifugation only one band was visible (Fig. 4B). We confirmed that the harvested viral DNA had the expected restriction maps with *Eco*RV, *Hin*dIII, and *Nhe*I digestion (Fig. 4A and C). Then we probed its Southern blot to an ITR probe. This should reveal any rearrangements at either the left or the right end of the genome that may account for the results or the contamination of the mutant virus stock with extraneous wild-type adenovirus. Because this analysis demonstrated that only two bands were detected in expected length in each restriction enzyme digestion, we could conclude that no rearrangement occurred (Fig. 4C). Then it was confirmed by Southern hybridization using λ-phage-specific probe that the purified virus had the expected deletion by Cre recombinase (Fig. 4D). Although we attempted to enhance the detection of the unrecombinated virus by PCR/Southern blot analysis, we could not detect it (Fig. 4E). It was obvious that the purified virus had the expected Cre-mediated deletion in the left end. Furthermore, we were able to exclude the possibility of a short sequence amplification by sequence analysis. The sequencing of the DNA from CsCI-purified virus revealed that the sequence of the left-end region is identical to that shown in Fig. 1b, except that the region between nt 192 and 358 is deleted and replaced by a single *lox*P site (data not shown). On the other hand, we did not detect any bands in the AdASw and AdBSw preparations because of inhibition of viral DNA encapsidation. These results prove that encapsidation of viral DNA with a deletion from nt 192 to nt 358 is possible without the amplification of the remaining packaging element.

Discussion

It has been shown that packaging of adenovirus DNA into empty capsids occurs with left-to-right polarity and the *cis*-acting elements necessary for packaging are located within the left-terminus region (Hammarskjold and Winberg, 1980; Hearing et al., 1987; Robinson and Tibbetts, 1984). Analysis of serial deletion and linker-scanning mutants in the left-terminus region showed that there are seven *cis*-acting elements, AI through AVII, each with different packaging efficacy (Grable and Hearing, 1990, 1992). Among these elements, AI, AII, AV, and AVI constitute the functionally dominant repeats. AI serves as the most efficient packaging domain in vivo, followed by AII and AV as an element with moderate activity, and AVI as the weakest packaging element. The other three elements, AIII, AIV, and AVII, have only minor effects on viral DNA packaging. These elements are functionally redundant and have an additive effect on the packaging efficiency. It has been shown that viral DNA carrying only AVI and AVII is nonviable, so the production of this mutant virus is not possible (Schmid and Hearing, 1997, 1998). However, in this study we demonstrated that a mutant adenovirus with a deletion of AI to AV is viable under our propagation conditions.

Because the Cre recombinase present in Cre-293 completely excises the DNA fragment between two *lox*P sites in each virus, only recombinant viral DNA is generated in Cre-293 cells (Fig. 2). When the packaged virus in Cre-293 cells is evaluated by plaque forming assays on 293 cells, the virus titers of AdASw and AdBSw are constant (10^6–10^7 PFU/ml). Because Cre-293 cells are so sensitive to mechanical manipulation that it is impossible to completely eliminate free virus particles from the medium by washing, titers

Table 1
Single-step growth of the recombinated adenovirus

	Input (PFU)	Yield (PFU)
Propagation 1 (P1)	9×10^5	3×10^6
Propagation 4 (P4)	4×10^7	3×10^8
Propagation 6 (P6)	4×10^7	2×10^8

Note. After determining the titers of the virus stocks at propagation 1, 4, and 6, these stocks were used for single-step growth curves. Each virus infected the 60-mm-diameter dish of 293 cells, and then the infectious virus yield at different time points was determined by plaque forming assay for 2 weeks of observation.

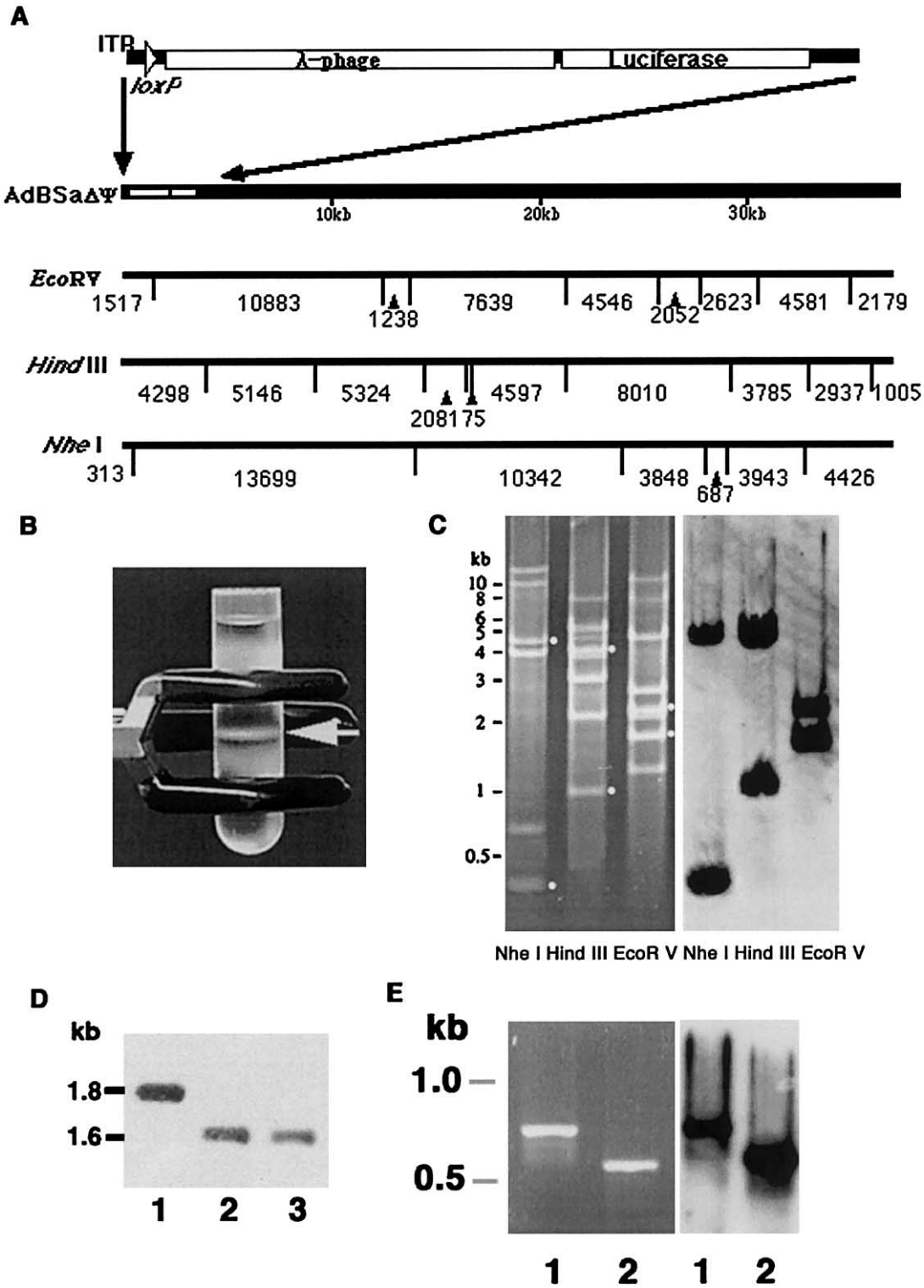

Fig. 4. Purification and structure of recombinant AdBSa (AdBSaΔψ). P6 lysate was propagated on 10^9 Cre-293 cells and purified by the conventional protocol. (A) Structure of recombinated AdBSa (AdBSaΔψ) in Cre-293 cells. Top line depicts the left-end region of recombinated AdBSa genome. *Eco*RV, *Hin*dIII, and *Nhe*I restriction maps, with fragment sizes indicated, are shown under the schematic linear maps. (B) After centrifugation, only one band (indicated by an arrow) was visible. A visible band was observed for AdBSa but neither AdASw nor AdBSw. (C) Left panel: Virus DNA was extracted from the purified virus, digested with *Eco*RV, *Hin*dIII, or *Nhe*I, and separated on agarose gel. All fragments corresponded to the expected fragments depicted in (A). Right panel: DNA from the agarose gel shown in left panel was transferred to a nylon membrane and hybridized with ITR probe depicted in Fig. 2A. Only two bands derived from right and left ITRs were visible in each restriction enzyme digestion. Because any another band could not be detected, any rearrangements at either the left or the right end of the virus genome did not occur. The white dots indicate the left or right end of the virus genome. (D) The left-end structure of the purified virus was determined by Southern blot analysis. Viral DNA from the purified virus was digested with restriction enzyme *Eco*RV and the

of less than 10^7 PFU/ml are likely to be due to free virus. We observed, however, that the titer of AdBSa rapidly increases after the fourth propagation. This high titer suggests that replication and packaging of AdBSa occurs even after recombination between the two *lox*P sites in Cre-293 cells. Actually, we observed that culture lysates from the second and third propagations of AdBSa made plaques of typical size and within the normal time, but lysates from the fifth and sixth propagations made smaller plaques at later times. That is to say, during the propagation of AdBSa the culture lysate contains two kinds of virus as mentioned above. Judging from the plots (Fig. 3), virus titer increases several times during the propagation sequence. Considering that wild-type adenovirus increases more than several thousand-fold in one infection (Graham and Prevec, 1991), the packaging efficiency of recombinant virus becomes less than 1% of that of wild-type adenovirus.

On the other hand, the fact that there was no difference in the virus titers of AdASw and AdBSw during propagation indicates that no packaging elements are located between nt 145 and nt 195. This observation is consistent with previous reports (Grable and Hearing, 1990). We were unable to detect any packaging ability of the fragment from nt 143 to nt 192 even under our propagation conditions. Furthermore, this observation suggests that the lambda, luciferase, or lox sites inserted into three kinds of viruses does not contribute essential nucleotides to re-create functional packaging elements. Besides this experimental evidence, we could not find the packaging consensus sequence in the inserted fragment by the homology rearch.

Although we demonstrated that AdBSa is able to proliferate in repetitive propagations even after the recombination event, it is still unclear whether a mutation occurs in the remaining packaging domain. To date, it has been observed that an adenovirus with poor packaging ability due to a deletion of the packaging domain can easily acquire an improved packaging ability by the multimerization of the remaining packaging elements (Schmid and Hearing, 1997). We attempted to purify each packaged adenovirus from large-scale cultures to analyze their structures. However, as we expected, it was impossible to purify packaged virus from AdASw or AdBSw large-scale cultures. Purification was possible only from AdBSa, and a purified band of packaged virus appeared after CsCI density centrifugation (Fig. 4B). Purified packaged virus had only one copy of the nt 358–454 region (Fig. 4D and E). Neither multimerization of the packaging *cis*-element nor any point mutations were detected by sequencing the left-terminus region. This indicates that viral DNA with a deletion from nt 192–358 is sufficient for stable viral viability under our propagation conditions. The Ad packaging consensus motif has been defined as a bipartite sequence with a conserved AT-rich and a GC-rich half site (5′-TTTGN_8CG-3′). Mutational studies have shown that the TTTG as well as the CG nucleotides are critical determinants for DNA packaging in vivo. Among the seven proposed *cis*-elements, only AI, AII, AV, and AVI have this consensus sequence, and actually, these four elements work as the major packaging elements in vivo. AVI, however, is unable to work as an independent packaging element in the absence of another major element. Only when at least one of the other major elements (AI, AII, or AV) is present in viral DNA can AVI improve packaging efficiency (Schmid and Hearing, 1997). Under the conditions used in our studies, however, the fragment from nt 358 to nt 455, which contains only AVI and AVII, is sufficient for viral DNA packaging. Probably only AVI, the weakest of the major packaging elements, is needed for packaging, because AVII is not a major element. The importance of AVI for packaging is obvious, because the deletion of nt 194 to nt 454 from the viral DNA by recombination (as in AdBSw) results in the complete loss of packaging ability.

In this study, using the Cre/*lox*P system we mapped the *cis*-elements required for packaging under specific conditions. These conditions do not exist in the natural world, however, but only during the preparation of HDAd. When HDAd is applied to human gene therapy, helper virus production must be kept as low as possible. The results presented here will be very useful in helper virus design. For example, even though Kochanek et al. used a mutant first-generation adenovirus SV5 with a deletion of AII–AV as a helper virus that resulted in a 1/90 encapsidation efficiency relative to wild-type Ad5, helper virus content was greater than that of HDAd after large-scale preparation (Kochanek et al., 1996). When we used AdASw as the helper virus to prepare large-scale HDAd on Cre-293 cells, we achieved efficient suppression of helper virus production to the extent that no helper virus band was visible after centrifugation.

Materials and methods

Construction of DNA

Plasmid pCI-neo-Cre was generated by the method described below. The Cre fragment was amplified from a nuclear localization signal (NLS)-tagged Cre-expressing adenovirus AxCANCre (Kanegae et al., 1995) (TaKaRa Biomedicals) by PCR using the following primer set: 5′-

left-end portion of the virus was detected with a λ-probe depicted in Fig. 2A. Lane 1: viral DNA from AdBSa. Lane 2: viral DNA from the purified virus after propagation on Cre-293 cells. Lane 3: viral and cellular DNA harvested at 48 h postinfection with Cre-293 cells. The result indicates that the left-end portion of the purified virus is shorter than that in AdBSa due to Cre-mediated excision. (E) Enhanced detection of unrecombinated virus DNA by PCR and Southern blot analysis. Left-end region was amplified and separated as mentioned in Fig. 2B, and then, the DNA was transferred to a membrane and probed with labeled ITR. Left panel is a result of PCR. Right panel is a result of Southern blot analysis. Lane 1 and lane 2 are AdBSa and the purified AdBSa in Cre-293, respectively. Any unrecombinated virus could not be detected even in this analysis.

ATGTCCAATTTACTGACCGT-3′, 5′-CTAATCGCCATCTTCCAGCA-3′. The product, which was deleted of the nuclear localization signal, was treated with T4-DNA polymerase and ligated to *Sma*I-digested pCI-neo (Promega).

Cells and generation of Cre-293 cells

The following cell lines were used: monkey kidney cells COS7 and human embryonic kidney cells HEK293 (American Tissue Culture Collection: ATCC). These cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Rockville, MD) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

To establish a stable cell line expressing the Cre recombinase, HEK293 cells were transfected at 80% confluence in a 100-mm-diameter dish with 30 μg pCI-neo-Cre DNA that had been linearized with *Bgl*II as a calcium phosphate precipitate. Forty-eight hours after transfection, cells were treated with trypsin and split at a ratio of 1:10. The resistant colonies were selected in DMEM supplemented with 10% FBS and 400 μg G418 (Sigma) per milliliter. Resistant colonies were subcloned in selective medium and tested for expression of functional Cre protein, resulting in the Cre-293 cell line.

Construction of viruses

Viruses were constructed by the COS/TPC method (Miyake et al., 1996). To construct three kinds of viruses containing *lox*P sites in various positions, the 42-kb cosmid pAdex1cw was manipulated. The 4.2-kb plasmid pAdexSal-self containing the adenovirus left end was generated first by self-ligation of pAdex1cw after *Sal*I digestion. Synthetic oligomers containing the *lox*P sequence and proper restriction sites were cloned into the *Afl*III (at nucleotide 143 from the adenovirus genome left end), *Bsr*GI (nt 192), *Sac*II (nt 358), or *Cla*I (nt 454) sites of pAdexSal-self. After these manipulations pAdex-ASw-lox-sal, pAdex-BSw-lox-sal, and pAdex-BSa-lox-sal were generated, which contain parallel *lox*P sites in the *Afl*III and *Cla*I, *Bsr*GI and *Cla*I, and *Bsr*GI and *Sac*II sites, respectively. From these plasmids three kinds of adenovirus left-end fragments containing *lox*P sites were harvested by *Eco*RI and *Swa*I digestion. Cosmids pAdex-ASw-lox, pAdex-BSw-lox, and pAdex-BSa-lox were generated by ligating each *Eco*RI -*Swa*I fragment and the 24-kb *Swa*I-*Eco*RI adenovirus genome fragment of pAdex1cw into *Eco*RI-digested pAdex1cw. We marked viruses by inserting the firefly luciferase gene in the E1 region. In addition, the 3.7-kb *Bsp*HI fragment of λ-phage was inserted in E1. For this purpose, pGL3-λluc generated by ligating the 3.7-kb blunt-ended *Bsp*HI fragment of λ-phage (nt 890 to nt 4650) into blunt-ended *Hin*dIII-digested pGL3-Control (Promega). Cosmids pAdex-ASw-lox-lucλ, pAdex-BSw-lox-lucλ, and pAdex-BSa-lox-lucλ were generated by ligating the 6.2-kb blunt-ended *Mlu*I-*Sal*I luciferase expression cassette and λ-fragment of pGL3-λ into *Swa*I-digested pAdex-ASw-lox, pAdex-BSw-lox, and pAdex-BSa-lox, respectively. These cosmids were sequenced intensively around the adenovirus left-end region and the sequences were verified.

One-half microgram of the *Eco*T22I-digested adenovirus genome covalently linked to the terminal protein and 14.5 μg of pAdex-ASw-lox-lucλ, pAdex-BSw-lox-lucλ, or pAdex-BSa-lox-lucλ were mixed. HEK293 cells in a 60-mm-diameter dish were transfected with the mixed DNA by the calcium phosphate method. After overnight transfection, the monolayer was overlaid with 0.5% agarose-containing culture medium. Recombinant viruses were purified and propagated according to standard procedures (Graham and Prevec, 1991).

Propagation of the virus

Confluent Cre-293 cells in a 60-mm-diameter culture dish were infected at a multiplicity of infection (m.o.i.) of 2 for 1 h with each kind of virus and cultured in 3 ml of medium for 3 days until a complete cytopathic effect (CPE) was observed. At this point, cells and medium were collected, and three rounds of freezing and thawing were carried out to prepare a crude lysate containing the packaged virus. The crude lysate from this infection step was named P1 (propagation 1). Two milliliters of P1 lysate was used to infect confluent Cre-293 cells in a 60-mm-diameter culture dish for 1 h, after supplementing with 1 ml of fresh medium. The cells were coinfected with a parental virus (AdASw, AdBSa, or AdBSw) at an m.o.i. of 2. P2 lysates were collected after the CPE. The same propagation procedures were repeated for another five propagations (P3–P7).

For large-scale preparations, three 60-mm-diameter dishes of Cre-293 cells were coinfected with 0.5 ml of P5 lysate and the parental virus supplemented with 2.5 ml fresh medium for each dish (P6). Finally, eight 150-mm-diameter dishes of Cre-293 (approximately 10^9 total cells) were infected with 1 ml of P6 lysate in 17 ml fresh medium. All other conditions were the same. The crude preparation was centrifuged to equilibrium on a CsCI buoyant-density gradient.

Determination of virus titer

To determine the virus titers after each propagation, lysates from the infected Cre-293 cells and the culture medium were prepared 48 h postinfection, and the amount of infectious virus was determined by plaque assays on 293 cells.

Southern blot analysis and PCR analysis of virus structure

Viral DNA was harvested by the standard method (Graham and Prevec, 1991). The left-end structure was determined by Southern blot analysis (Sambrook, 1989) and

PCR. Restriction enzyme-digested viral DNA was transferred from an agarose gel to a nylon membrane (Hybribond-N, Amersham) by capillary transfer, hybridized to alkaline phosphatase-labeled DNA probes, and visualized by a chemiluminescent reaction as specified by the manufacturer (CDPstar, Boehringer Mannheim). For the PCR analysis ITR-primer (5′-GCCAATATGATAATGAGGGG-3′) and λ-primer (5′-GGCAGACTTCACCACATTCA-3′) were used.

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